Regulation of hydrophobin genes in Schizophyllum commune

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Regulation of *SC3* expression in *Schizophyllum commune*

In the homobasidiomycete *Schizophyllum commune* four hydrophobin genes have been identified. Hydrophobins are small secreted proteins, having identical spacing of eight cysteine residues and share a common hydropathy pattern (for review see Wessels, 1997). The *SC1*, *SC4* and *SC6* hydrophobin genes are specifically transcribed in the *MAT*A≠ *MAT*B≠ heterokaryotic secondary mycelium and their transcripts can accumulate to high extent during fruiting body formation (Mulder and Wessels, 1986; Wessels *et al.*, 1995). However, the *SC3* hydrophobin gene, is highly expressed in both the monokaryon (primary mycelium) and the dikaryon (secondary mycelium) during formation of aerial hyphae (Schuren and Wessels, 1990; Schuren *et al.*, 1993c). The SC3 hydrophobin is secreted at the tip of the aerial hyphae and by interfacial self-assembly it coats these hyphae with an SDS-insoluble hydrophobin membrane that makes them highly hydrophobic (Wösten *et al.*, 1993; 1994b). A strain containing a targeted disruption of the *SC3* gene was impaired in the formation of aerial hyphae. Under certain growth conditions aerial hyphae could nevertheless form but these appeared to be hydrophilic (Wösten *et al.*, 1994a; van Wetter *et al.*, 1996).

Run-on experiments have shown that expression of the hydrophobin genes from *S. commune* are regulated at the level of transcription (Schuren *et al.*, 1993c). *SC3* expression is regulated by the *THN* gene; in a monokaryotic *thn* mutant, *SC3* is suppressed while few aerial hyphae form (Wessels *et al.*, 1991b). In addition, *SC3* expression is regulated by the *MAT*B mating-type genes as well. In a common-*MAT*A heterokaryon (*MAT*A= *MAT*B≠, resulting in a *MAT*A-off *MAT*B-on phenotype) and a homokaryotic strain carrying a constitutive mutation in the *MAT*B mating type gene, *SC3* expression is suppressed (Ásgeirsdóttir *et al.*, 1995; Ruiters *et al.*, 1988). In both cases *SC3* suppression is associated with poor formation of aerial hyphae leading to a flat morphological appearance.

To study the regulatory sequences of *SC3*, the availability of a reporter gene and a targeting system, to direct introduced DNA to certain loci, would be convenient. However, in *S. commune* most foreign genes are not expressed and targeting by homologous integration seems to be a rare event. Chapter 2 shows that an alternative system could be used based on the introduction of the *SC3* gene (with 1250 bp upstream sequences) into an *SC3*-disrupted strain (*SC3::phleo*^{r}, *ura1*) by cotransformation with the wild-type *URA1* gene. To counteract the unavailability of a targeting system, a number of transformants was analysed for recovery of *SC3* expression. Restoration of *SC3* expression, although generally very poor, could be detected on both RNA and protein levels leading to recovery of formation of hydrophobic aerial hyphae. To improve the number of transformants with good recovery of *SC3* use was made of a phleomycin resistance gene as the selective marker present on the same vector as the *SC3* genomic fragment.

Since the *SC3* disrupted strain already contains a phleomycin resistance cassette, selection was performed at an elevated phleomycin concentration and caffeine was added to make the cells more susceptible to phleomycin. This indeed resulted in much better recovery of *SC3* expression. As in the co-transformation experiments, Southern blot analysis showed no correlation between *SC3* copy number and the level of *SC3* expression therefore suggesting position-dependent expression of integrated DNA. However, regulation of the introduced *SC3* gene was as the original endogenous gene indicating that all *cis*-regulatory sequences were present on the introduced *SC3* genomic fragment. To further examine the upstream region of the *SC3* gene, plasmids containing the *SC3* gene with 5' promoter deletions were made. Chapter 3 shows that introduction of these plasmids in the *SC3* disrupted strain resulted in the identification of a positively acting element in between 952 and 627 bp upstream of the transcription start-point of the *SC3* gene. When representatives of these transformants were mated with a strain that contains a different *MAT*B but the same *MAT*A gene (a common-*MAT*A interaction) down-regulation of *SC3* by the *MAT*B genes could be studied. Results indicated that the regulatory sequences necessary for *SC3* suppression are not present in the -1264 to -952 region but may occur anywhere between -952 bp and the transcription start-site. Sequence analyses of the upstream -952/-627 sequence revealed the presence of some direct and inverted repeats. The inverted repeat is part of a sequence resembling the APE element (Carattoli *et al.*, 1994) identified upstream of the *al-3* gene and found in front of other light-induced genes of *Neurospora crassa* including the *eas* gene coding for a fungal hydrophobin involved in the coating of the conidial surface belongs to (Lauter *et al.*, 1993; Bell-Pedersen *et al.*, 1993). Another interesting motif found in the -952/-627 sequence resembles the BRE element identified in *Aspergillus nidulans* (Chang and Timberlake, 1993) upstream of genes regulated by the regulatory gene *brlA*. *RodA*, which codes for a hydrophobin that is found on conidia (Stringer *et al.*, 1991), is positively regulated by *brlA* and repeats of the BRE element are found in its upstream sequence. More putative BRE elements were found in front of the *SC3* gene and additionally in the upstream sequences of the dikaryon-expressed genes (*SC4*, *SC7* and *SC14*) as well. These sequences might therefore act as targets for a regulatory gene like the *THN* gene that was found to regulate (directly or indirectly) expression of all these genes (Wessels *et al.*, 1991b).

Furthermore a motif, that is recognised by a subfamily of HMG-box containing proteins (Laudet *et al.*, 1993) was identified at position -173. Proteins that belong to this group are Ste11 of *Schizosaccharomyces pombe* (Aono *et al.*, 1994) and a protein that recognizes the pheromone responsive element (PRE) of *Ustilago maydis* (Hartmann *et al.*, 1996; Urban *et al.*, 1996). In both organisms these elements confer pheromone-dependent activation of gene expression. The presence of such an element in front of the *SC3* gene which is down-regulated by the *MAT*B

genes (coding for pheromone and pheromone receptor proteins, Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997), might therefore be a significant observation.

Homology-dependent silencing of the *SC3* gene

Hydrophobins display some special features, like self-assembly at hydrophilic/hydrophobic interfaces and coating of hydrophobic materials which make them interesting molecules for technical and medical applications. The SC3 hydrophobin is abundantly secreted into the culture medium from which it can be purified rather easily (Wessels, 1997). To improve the yield of purified SC3, multiple copies of the *SC3* gene containing all *cis*-regulatory sequences necessary for proper expression were introduced into the wild-type strain of *Schizophyllum*. Surprisingly, these attempts resulted in silencing of the introduced and endogenous *SC3* gene-copies in almost 90% of the transformants (Chapter 4). The resulting absence of SC3 secretion led to a wettable phenotype; aerial hyphae lacked SC3 protein and were therefore hydrophilic. Nuclear run-on experiments revealed that *SC3*-silencing occurred at the level of transcription and was presumably mediated by cytosine methylation of genomic DNA. This was shown by isoschizomeric analyses and the use of 5-azacytidin (a compound known to prevent cytosine methylation) during growth which resulted in suppression of silencing. Southern analyses localised the observed cytosine methylation primarily in the coding region of the *SC3* gene-copies (Chapter 5). Involvement of cytosine methylation was further demonstrated by analysing the phleomycin resistant progeny from a cross between an *SC3*-silenced and a compatible wild-type colony. It was found that most of these siblings still displayed *SC3*-silencing but in some recovery of *SC3* expression was observed. Southern analyses revealed that the latter type of colonies still contained all introduced *SC3* gene copies but the intensity of methylation of these particular sequences had clearly decreased. It is thus apparent that methylation plays a role in at least the maintenance of the silenced state of the *SC3* gene copies. In agreement with this were the results obtained in experiments in which we analysed whether silencing could be transmitted to other nuclei when they share the same cytoplasm. In this heterokaryotic mycelium (two nuclei per hyphal cell), containing an *SC3*-silenced and a non-transgenic wild-type nucleus, silencing was not introduced in the wild-type nucleus, which is consistent with methylation being involved in maintenance of *SC3*-silencing.

Well studied gene inactivations of duplicated sequences in fungi that involve intensive cytosine methylation are RIP (repeat induced point mutation) in *Neurospora crassa* (Selker *et al.*, 1987) and MIP (methylation induced premeiotically) in *Ascobolus immersus* (Goyon and Faugeron, 1989). In both processes cytosine residues in the entire homologous sequence can become methylated whereas in the case of RIP this is followed by C to T point mutations.

Methylation being present in the entire homologous sequence was also observed in plants (Assaad *et al.*, 1993), although transcriptional gene silencing in these organisms seems mostly associated with methylation of promoter sequences (Matzke *et al.*, 1989; Meyer *et al.*, 1993;). Although methylation was mainly believed to be associated with transcriptional gene silencing, it has now also been observed in the 3'-end of the coding region in some cases of post-transcriptional gene-silencing (Ingelbrecht *et al.*, 1994; English *et al.*, 1996). Our results clearly show that silencing of the *SC3* gene occurs at the transcriptional level and is associated with methylation particularly in the coding region of the homologous sequences. It is therefore not unlikely that transcription is still initiated but that the elongation of the transcripts is hampered due to the presence of methylation in the coding sequence. The formation of truncated aberrant RNAs could have gone undetected in the nuclear run-on assays because these molecules were possibly too small. Additional nuclear run-on experiments and/or RNase protection assays might further elucidate this enigma.

Although our results suggest that maintenance of homology-dependent silencing of the *SC3* gene is mediated by methylation of the homologous *SC3* sequences, it is yet uncertain how *de novo* methylation and the resulting gene-silencing are induced. Matzke and Matzke (1995) proposed that three possibilities can be distinguished: methylation as a response to foreign DNA, DNA-DNA pairing, or a DNA-RNA interaction. The first possibility seems unlikely since introduction of other plasmids into the wild-type strain of *S.commune* did not result in methylation and inactivation. More important, of the whole introduced sequence (pXGPhT-S3) only the coding region of the *SC3* gene became detectably methylated. No other sequences seemed to be methylated and all the transformed strains still transcribed the phleomycin resistance gene.

The second possibility that *SC3*-silencing is based on recognition of homologous sequences via DNA-DNA interactions cannot entirely be excluded. This model is derived from the RIP process found in *Neurospora* and the MIP process in *Ascobolus*, where DNA-DNA interactions act in a pairwise manner (Selker and Garrett 1988; Fincham *et al.*, 1989; Rossignol and Faugeron, 1994) leading to *de novo* methylation which in the case of RIP is followed by C to T point-mutations. A characteristic feature of RIP and MIP is that it acts in principal on every duplicated sequence present in the genome on condition that the sequence homology is large enough (a few hundred base pairs for linked duplications; Selker, 1997). However, this is definitely not the case for *SC3* silencing; only *SC3* fragments containing a full length promoter sequence are able to induce silencing at high frequency (Chapter 4). Moreover, until now silencing has only been found for the *SC3* gene whereas introduction of other (homologous) sequences was apparently unable to trigger silencing (T.A. Schuurs, unpublished data). Recent

results (Stam *et al.*, 1997b) show that even for some cases of post-transcriptional gene silencing the way in which the introduced DNA is integrated is important; silencing of chalcone synthase genes in Petunia appeared to be associated with the presence of inverted transgene repeats. However, the structure of integrated DNA seems unimportant in *SC3* silencing since silencing is observed in single copy transformants as well (Chapter 4 and 6).

In the DNA-RNA model (over)production of RNA would lead to *de novo* DNA methylation by formation of a DNA-RNA hybrid which could serve as a template for DNA-methylases. The first report showing that a DNA-RNA association can serve as a signal for *de novo* methylation came from Wassenegger *et al.* (1994) who showed *de novo* DNA methylation of viroid cDNA sequences only after replication and formation of RNA molecules had occurred. Several results obtained during the present study are pointing to existence of such a DNA-RNA model in the case of *SC3* silencing in *S. commune*. Firstly, silencing during growth was always unidirectional; silencing took place after expression of *SC3* had occurred (Chapter 4) and could then be stably transmitted during subculturing. Secondly, removal of the promoter sequence had a severe effect on the incidence of silencing; only 7.5% of the transformants exhibited *SC3* silencing which mostly occurred in sectors (and not in the entire colony) which is the less severe type of silencing (Chapter 4) whereas introduction of the entire *SC3* genomic sequence (including the promoter) normally resulted in silencing of almost 90% of the transformants. Removal of a smaller upstream sequence known to be necessary for *SC3* transcription (plasmid pPS3-ph, see Chapter 3) had the same effect on the occurrence of silencing (T.A. Schuurs, unpublished data). Furthermore, in Chapter 2 we showed that if the *SC3*-disrupted strain was transformed with a *SC3*-gene containing plasmid by cotransformation then complementation of *SC3* expression in these tranformants was very poor (although one or more *SC3* gene copies were integrated). This complementation system very much improved after we placed the phleomycin resistance cassette and the *SC3* gene fragment on the same plasmid (Chapter 2). If the wild-type strain containing an intact copy of the *SC3* gene was transformed with the latter plasmid gene silencing was observed in about 90% of the transformants (Chapter 4). However, if we introduced the same *SC3*fragment in this wild-type strain by cotransformation, silencing was rarely observed although the transformants contained extra *SC3* gene copies (T.A. Schuurs, unpublished results). All these results together suggest that the mere presence of duplicated sequences is not sufficient to trigger silencing of the *SC3* gene. Apparently, other factors are also required, of which *SC3* transcription is a likely candidate (Fig. 1). In this model (over)production of *SC3* mRNA would lead to *de novo* DNA methylation by formation of a DNA-RNA hybrid which could serve as a template for DNA-methylases (Matzke and Matzke, 1995). Our results localising methylation in the coding region of the *SC3* gene only is therefore an interesting observation and consistent with this

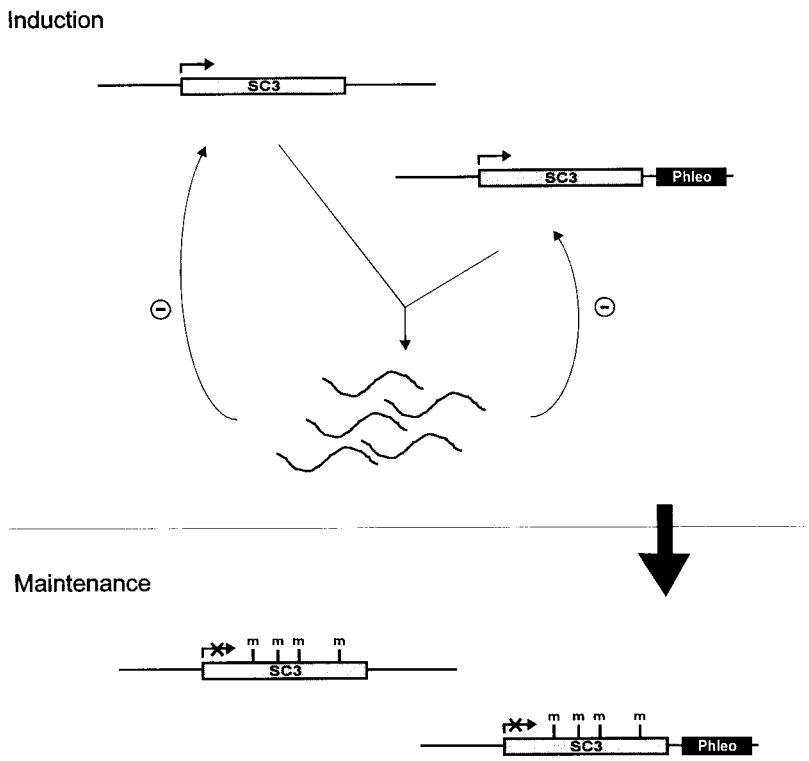

Figure 1: *Model for induction and maintenance of homology-dependent silencing of the SC3 gene in Schizophyllum commune. See text for detailed description.*

RNA-DNA silencing model in which the RNA itself is the messenger and initiator of silencing, while methylation may be involved in the maintenance of gene inactivity.

Transformants, still having *SC3* expression, were further analysed for presence of elevated levels of SC3 secretion (Chapter 6). Indeed, some of these transformants showed increased levels of SC3 secretion reaching levels of two to three times that of wild-type. Southern analyses revealed that these (over)producing transformants contained only one or two copies of the introduced plasmid whereas *SC3* silencing was generally associated with multi-copy

transformants, although silencing was observed in single copy integrants as well (Chapter 4 and 6). The observation that only low copy number transformants are able to secrete SC3 leads us to conclude that extreme overexpression of the *SC3* gene will never be reached by introducing multiple-copies of the gene. Selecting for *S. commune* strains that are impaired in *SC3* silencing is one way of dealing with this problem. Heterologous expression of *SC3* in organisms like *Aspergillus niger* is another. The latter approach is currently undertaken in our lab.

Positioning of nuclei and differential gene expression

When two compatible monokaryons containing different *MAT*A and *MAT*B genes make contact, hyphae may fuse and a heterokaryotic dikaryon (with two closely associated compatible nuclei per hyphal compartment) is formed that will eventually develop fruiting bodies under the right environmental conditions. Among the genes that are specifically expressed in a *MAT*A≠ *MAT*B≠ dikaryon (secondary mycelium) of *Schizophyllum*, are the *SC4* and *SC7* genes. Their mRNAs can accumulate to high levels during fruiting-body formation (Mulder and Wessels, 1986) in which the SC4 hydrophobin provides air channels with a hydrophobic lining (Wessels *et al.*, 1995). Immunolabelling revealed the presence of SC7 specifically in the extracellular matrix of fruiting-body hyphae (Ásgeirsdóttir *et al.*, 1995). Both proteins are also secreted by submerged dikaryotic vegetative hyphae into the culture medium. The *SC3* hydrophobin gene is expressed during formation of aerial hyphae in both mono- and decurion. As mentioned before, *SC3* expression is down-regulated by the *MAT*B genes; in a common-*MAT*A heterokaryon (*MAT*A= *MAT*B≠) and a homokaryotic strain carrying a constitutive mutation in the *MAT*B gene (*MAT*A41 *MAT*Bcon), *SC3* is suppressed and few aerial hyphae are formed (Ásgeirsdóttir *et al.*, 1995). Down-regulation of *SC3* by the presence of different *MAT*B genes seems to contradict the observation that in a *MAT*A≠ *MAT*B≠ heterokaryon *SC3* expression does occur in spite of the presence of different *MAT*B genes (Wessels *et al.*, 1987). The finding of disruption of the binucleate state (that is the appearance of a considerable nuclear distance between the two compatible nuclei in a hyphal compartment) in incipient aerial hyphae of the *MAT*A≠ *MAT*B≠ heterokaryon, led to the hypothesis (Ásgeirsdóttir *et al.*, 1995) that close spatial association of the two compatible nuclei is necessary for proper interaction of the mating-type genes to occur. Indeed, in Chapter 7 it was shown that binucleate hyphae with juxtaposed nuclei secrete SC4 and SC7, while SC3 appeared to be absent. Certain growth conditions (shaken suspension cultures or growth on hydrophobic substrates or growth in the dark) disrupted the binucleate state in that the compatible nuclei became separated at a considerable distance. Under these conditions SC4 and SC7 were not secreted while SC3 was secreted to a high degree (Chapter 7). Apparently, when the nuclei are in close proximity the dikaryon-expressed genes are switched on by interaction of the products

of the *MAT*A and *MAT*B mating-type genes, while *SC3* is suppressed by interacting products of the *MAT*B genes, as occurs in the common-*MAT*A heterokaryon (*MAT*A= *MAT*B≠). Growth conditions that lead to disruption of the binucleate state apparently result in abolishment of interaction between *MAT*B mating-type proteins. Under these conditions transcription of the dikaryon-specific genes in the *MAT*A≠ *MAT*B≠ heterokaryon is not activated at all, while in the absence of a *MAT*B interaction *SC3* is expressed and aerial hyphae are formed.

Since it is the action of the *MAT*B mating-type genes that down regulates *SC3* expression, involvement of these mating-type genes is implied in the proposed model in which differential gene expression is determined by the distance of the two compatible nuclei in the secondary mycelium of *S. commune*. The *MAT*B mating-type genes have now been shown to encode pheromones and pheromone receptors (Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997) and signalling between the two nuclei may be mediated by this system; compatible nuclei positioned in close proximity allow for in an interaction of pheromones encoded in one nucleus with the pheromone receptor of the other nucleus and *vice versa*. If the nuclei are positioned at a considerable distance from each other this pheromone/receptor interaction would be lost resulting in a failure to activate the dikaryon-expressed genes (e.g. *SC4* and *SC7*) and loss of suppression of genes involved in the typically monokaryotic type of growth (e.g. *SC3*). Existence of such a model requires the *MAT*B mating type gene products to be synthesized and located in a cytoplasmic domain specifically around the nucleus in which they are transcribed. The site of action of the compatible pheromone/pheromone receptor interaction can be either intra- or extracullular. If signalling occurs within the cytoplasm it could be that the pheromone receptors are located on the nuclear membrane of the nucleus in which they were transcribed. In this model the pheromone is present in a concentration gradient diffusing away from the nucleus. Only if the two compatible nuclei are in close proximity a pheromone/pheromone receptor interaction is able to take place. A variant to this model localises the pheromone receptor in the plasma membrane and the pheromone primarily in the cell wall. In this model localisation of these factors is again determined by their synthesis in a cytoplasmic domain around the nucleus in which they are encoded resulting in a concentration gradient of the pheromone receptor in the plasma lemma and of the pheromone in the cell wall in the direct vicinity of the nucleus. Extracytoplasmic signalling now occurs if the nuclei are in close proximity and the resulting concentration gradients in plasma lemma and cell wall overlap each other (Fig. 2). Localisation of pheromone receptor in the plasma lemma as proposed in the latter model is more consistent with observations made in other fungi like for example *S. cerevisiae* (Burkholder and Hartwell, 1985) and *U. maydis* (Bölker *et al.*, 1992). However, the pheromone/pheromone receptor system in these fungi is involved in attraction between and subsequent fusion of compatible mates which seems not to

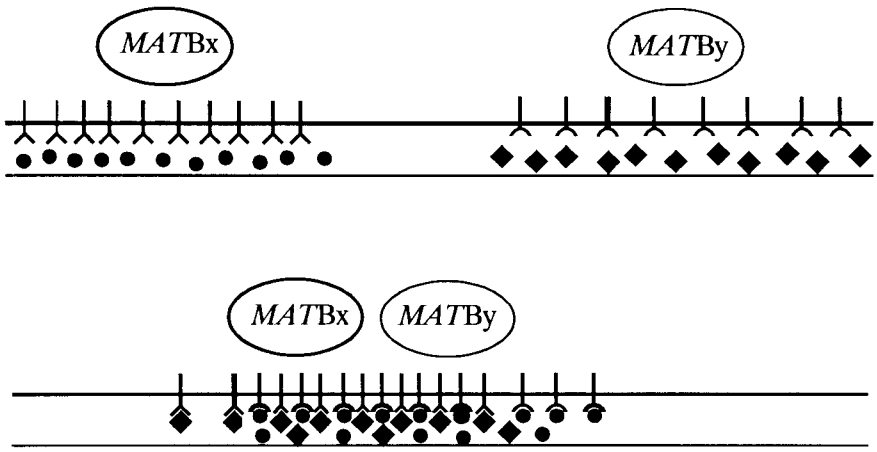

Figure 2: *Model explaining differential gene expression in the secondary mycelium by means of changing the distance between the two compatible nuclei in a hyphal cell compartment. The MATB encoded pheromone receptor is located in the plasma lemma while the pheromones are present in the cell wall matrix due to 'targeted' secretion via ABC-transporters. See text for detailed explanation.*

be the case for *S. commune* since fusion of hyphae occurs regardless their mating-type specificity. This would be consistent with the model in which the pheromones are primarily present in a cell wall domain and are not secreted into the medium. The *MAT*B encoded pheromones do not contain signal sequences and secretion likely occurs via ABC transporters like it is the case for the **a**-factor of *S. cerevisiae* (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). Secretion mediated by these ABC transporters could very well target the pheromones toward the cell wall surrounding the cytoplasmic domain dominated by the nucleus in which they were transcribed.

Which model ultimately will prevail is dependent on the outcome of experiments aimed at determining the exact localisation of the *MAT*B encoded pheromones and pheromone receptors in *S. commune*. Whatever the results will be, regulation of gene activity by modulating the distance between haploid genomes may represent a regulatory mechanism unique to fungi that maintain heterokaryotic cells rather than forming diploids as in plants and animals. It apparently provides for additional regulatory possibilities not encountered in haploid/diploid organisms which could make them more flexible in adapting to changing environmental conditions.